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Ultrastructure of *Isospora suis* during excystation and attempts to demonstrate extraintestinal stages in mice

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ABSTRACT

Pinckney, R.D., Lindsay, D.S., Toivio-Kinnucan, M.A. and Blagburn, B.L., 1993. Ultrastructure of *Isospora suis* during excystation and attempts to demonstrate extraintestinal stages in mice. *Vet. Parasitol.*, 47: 225–233.

Transmission electron microscopy was used to examine the structure of the oocysts, sporocysts and sporozoites of *Isospora suis* during in vitro excystation. Oocysts were ground in a teflon-coated tissue grinder to free most sporocysts and to allow for exposure of oocysts and sporocysts to excystation medium. The suspension of oocysts and sporocysts was incubated at 37°C for 0–45 min in excystation medium. After incubation, the intact oocysts and sporocysts, excysted sporocysts, and sporozoites in the excystation medium were pelleted by centrifugation and fixed for transmission electron microscopy. The oocyst wall was composed of three layers. Treatment with 1.5% (v/v) sodium hypochlorite solution removed the outer layer. The sporocyst wall was composed of two layers, the inner layer of which was interrupted by sutures. During excystation these sutures separated, allowing release of the sporozoites. Sporozoites were elongate and possessed all of the organelles typical of coccidial sporozoites. Tissues from experimentally inoculated outbred Swiss–Webster or inbred BALB/c mice were examined for extraintestinal stages (monozoic cysts) of *I. suis* by immunoperoxidase staining using specific antisera. Extraintestinal stages were not observed in mice, including those given methylprednisolone acetate.

INTRODUCTION

Frenkel (1977) proposed that the species of *Isospora* infecting dogs and cats be placed in a genus (*Cystoisospora*) different from the traditional *Isospora* species that are confined to a host's intestinal tract because these parasites could form extraintestinal stages (monozoic cysts) in both definitive

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and paratenic hosts (Frenkel and Dubey, 1972; Dubey, 1975, 1978, 1979; Dubey and Mehlhorn, 1978). Dubey (1977) came to the same conclusion independently and proposed the genus *Levineia* for the *Isospora* species that produce extraintestinal stages in definitive and paratenic hosts. Box et al. (1980) recognized the similarities in the excystation processes of *Toxoplasma gondii*, *Sarcocystis* species, and the *Isospora* species infecting dogs and cats and concluded that the lack of a Stieda body in sporocysts correlated with certain features of the life cycles of these parasites. *Isospora* species that excysted by release of sporozoites through a Stieda body were believed to be monoxenous, while those that excysted by collapse of sporocyst wall plates to release sporozoites were believed to be heteroxenous. They believed that these observations supported Frenkel's (1977) taxonomic classification. Box et al. (1980) suggested that the name *Isospora* be retained for coccidia with disporocystic oocysts and sporocysts with a Stieda body and these coccidia be placed in the family Eimeriidae, and that the disporocystic coccidia without Stieda bodies be placed in the family Sarcocystidae.

Isospora suis is an economically important cause of intestinal disease in nursing pigs (Stuart et al., 1978, 1980). Nursing pigs become infected even though sows apparently seldom excrete *I. suis* oocysts in their feces (Vetterling, 1966; Greiner et al., 1982; Lindsay et al., 1984; Stuart and Lindsay, 1986). Oocysts of *I. suis* are disporocystic and lack a Stieda body. This structural relationship to other mammalian *Isospora* species suggests that extraintestinal stages should be present in the *I. suis* life cycle. Extraintestinal stages, if present, may be important in the epidemiology of *I. suis* infections.

To better characterize the biology of *I. suis*, we used transmission electron microscopy to examine the excystation process of *I. suis*, and immunohistological methods to examine the tissues of experimentally inoculated mice to determine if extraintestinal stages of *I. suis* were present in their tissues.

MATERIALS AND METHODS

Preparation of oocysts, sporocysts, and sporozoites

Isospora suis oocysts were isolated and concentrated from the feces of experimentally infected, weaned pigs. Oocysts were sporulated in 2.5% (w/v) potassium dichromate solution, cleaned of fecal debris and enumerated using a hemacytometer. Oocysts were stored in 2.5% potassium dichromate solution at 4°C for up to 60 days before use.

Oocysts were washed free of potassium dichromate solution by centrifugation and resuspended in Hank's balanced salt solution (HBSS). To determine the effects of sodium hypochlorite (SH) (Clorox®, Oakland, CA) solution on the oocyst wall, some oocysts were incubated in a 1.5% (v/v) SH solution in an ice bath for 10 min, then washed in HBSS by centrifugation to remove

the SH solution. Samples of oocysts that were SH-treated or received no SH treatment were ground in a teflon-coated tissue grinder in an ice bath, to free most sporocysts and to allow for exposure of inner oocyst walls and sporocysts to excystation medium. The sporocyst/oocyst suspension was incubated in excystation medium consisting of 0.75% (w/v) sodium taurocholate and 0.25% (w/v) trypsin in HBSS at 37°C for 30–45 min. For some preparations, excysted sporozoites were separated from oocyst walls and empty sporocysts by fiber-column filtration (Bontemps and Yvore, 1974).

Transmission electron microscopy

Samples were fixed in buffered 3% glutaraldehyde, post-fixed in 1% (w/v) osmium tetroxide in Millonig's phosphate buffer (pH 7.3), dehydrated in a graded series of ethanol, and embedded in Spurr's resin. Sections were stained with uranyl acetate and lead citrate and examined using a Philips 301 transmission electron microscope operating at 60 kV.

Examination of mice for extraintestinal stages of I. suis

Seven female outbred Swiss–Webster (SW) mice weighing 18–22 g, and four female BALB/c mice weighing 18–22 g were used for studies on extraintestinal stages. Some mice were inoculated intramuscularly with 4 mg of methylprednisolone acetate (MPA) (Upjohn, Kalamazoo, MI). The MPA was given in hopes of enhancing infections because severe extraintestinal isosporiasis has been reported in humans with acquired immune deficiency syndrome and *Isospora belli* infection (Restrepo et al., 1987). The protocol for inoculation and examination of the mice is given in Table 1. Tissues collected and fixed in neutral buffered formalin from each mouse at necropsy were brain, tongue, heart, thigh muscle, thymus, lungs, liver, spleen, pancreas, kidneys, adrenals and mesenteric lymph nodes (MLN). A portion of MLN from each mouse was examined as an unstained fresh squash preparation with Normarski interference–contrast (NIC) microscopy. Formalin fixed tissues were embedded in paraffin, sectioned at 8 μm, and stained with hematoxylin and eosin. Duplicate sections were left unstained and used in an avidin–biotin peroxidase complex (ABPC) immunohistologic test.

The ABPC test was performed as described (Lindsay and Dubey, 1989) using rabbit anti-*I. suis* antiserum. The serum had a titer of 1:400 to *I. suis* sporozoites in an indirect immunofluorescent antibody (IFA) test and was used at a dilution of 1:200 in the ABPC test. This antiserum reacted with Type 1 meronts/merozoites of *I. suis* in the intestines of experimentally infected pigs using the ABPC test.

Serum samples were collected from the BALB/c mice at necropsy and examined in an IFA test against *I. suis* sporozoites. Serum samples from four

TABLE 1

Protocol for inoculation, methylprednisolone acetate (MPA) treatment, and examination of 11 mice for extraintestinal stages of *Isospora suis*

Mouse strain	Dosage[1]	MPA[2]	Day PI[3]	Results[4]
Swiss–Webster	285/360	No	28	Negative
		Yes	28	Negative
		No	35	Negative
		Yes	35	Negative
		No	42	Negative
		Yes	42	Negative
		No	49	Negative
BALB/c	0/500	No	22	Negative
	0/250	Yes	22	Negative
	0/500	No	55	Negative
	0/250	Yes	55	Negative

[1]Number of orally inoculated *I. suis* sporocysts/oocysts $\times 10^3$.
[2]Swiss–Webster mice received 4 mg MPA 7 days prior to and on the day of sporocyst/oocyst inoculation; BALB/c mice received 4 mg MPA on the day of inoculation.
[3]Day post-inoculation when mice were examined at necropsy.
[4]Results of examination of tissues for extraintestinal stages.

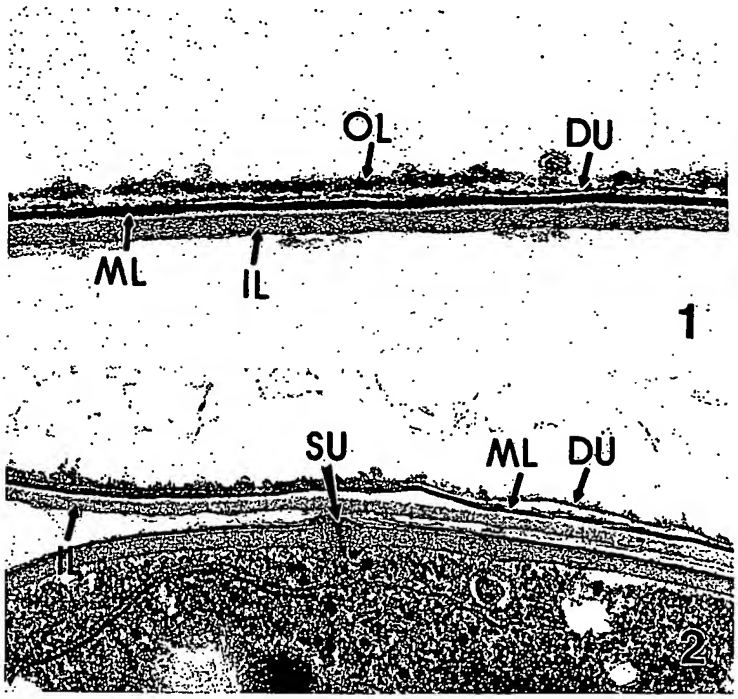
mice not infected with *I. suis* and a serum sample from a mouse with an IFA test titer of at least 1:800 to *T. gondii* served as controls. Sera were examined at doubling dilutions from 1:50 to 1:800.

To determine if *I. suis* undergoes excystation in mice, two female SW mice were orally inoculated with 6×10^5 *I. suis* oocysts, and examined 4 and 6 h post-inoculation (PI). Smears of luminal contents were made from the stomach, duodenum, jejunum, ileum and cecum of these mice, and examined with NIC microscopy for excysted oocysts and sporozoites. A portion of MLN was also examined with NIC microscopy for *I. suis* sporozoites.

RESULTS

The oocyst wall was composed of three layers (Fig. 1). The outer layer was 28–42 nm thick, irregular, electron-dense, and osmiophilic. An ill-defined double unit membrane complex separated the outer layer from the middle layer. The middle layer was 7–28 nm thick, homogeneous, electron-dense, and osmiophilic. The inner layer was 28–42 nm thick, homogenous, and electron-lucent. The outer layer and double unit membranes were usually removed by exposure to 1.5% SH solution, although the double unit membrane complex and patches of the outer layer were present on some oocyst walls that had been exposed to SH solution (Fig. 2).

The sporocyst wall was composed of two layers (Figs. 2 and 3) and was 63–84 nm thick, except in areas of sutures where it was thickened to 113–134



Figs. 1 and 2. Transmission electron micrographs of the oocyst wall of *Isospora suis*. Fig. 1. Oocyst wall with outer layer (OL), double unit membrane complex (DU), middle layer, and inner layer (IL), magnification×98 560. Fig. 2. Oocyst wall previously treated with sodium hypochlorite solution. The outer layer is removed leaving the double unit membrane complex (DU), middle layer (ML), and inner layer (IL) of the oocyst wall. Note that a sporocyst wall and suture (SU) are present beneath the inner layer of the oocyst wall. Magnification×41 580.

nm. The outer layer was 14–28 nm thick and enclosed the entire sporocyst. The inner layer was 49–56 nm thick and was interrupted by electron-dense, osmiophilic sutures that were confined to the inner layer which was enlarged to 85–100 nm in this area. A central electron-dense, osmiophilic core that was 77–85 nm × 14–42 nm in size was present in each suture. During excystation the central core apparently dissolved and the opposing plates separated. The sporocyst wall then curved inward on itself, releasing the enclosed sporozoites.

Sporozoites were elongate and contained all the organelles typical of coccidial sporozoites. The nucleus was in the central or posterior region of the sporozoite. Rhoptries and micronemes were abundant and were usually present anterior to the nucleus. Crystalloid bodies were present, but refractile bodies were not observed in sporozoites.

Evidence of excystation was observed in the mice examined 4 and 6 h PI of *I. suis* oocysts. Sporulated oocysts, excysted sporocysts within oocysts, and free sporocysts were observed in the duodenum, jejunum, ileum and cecum

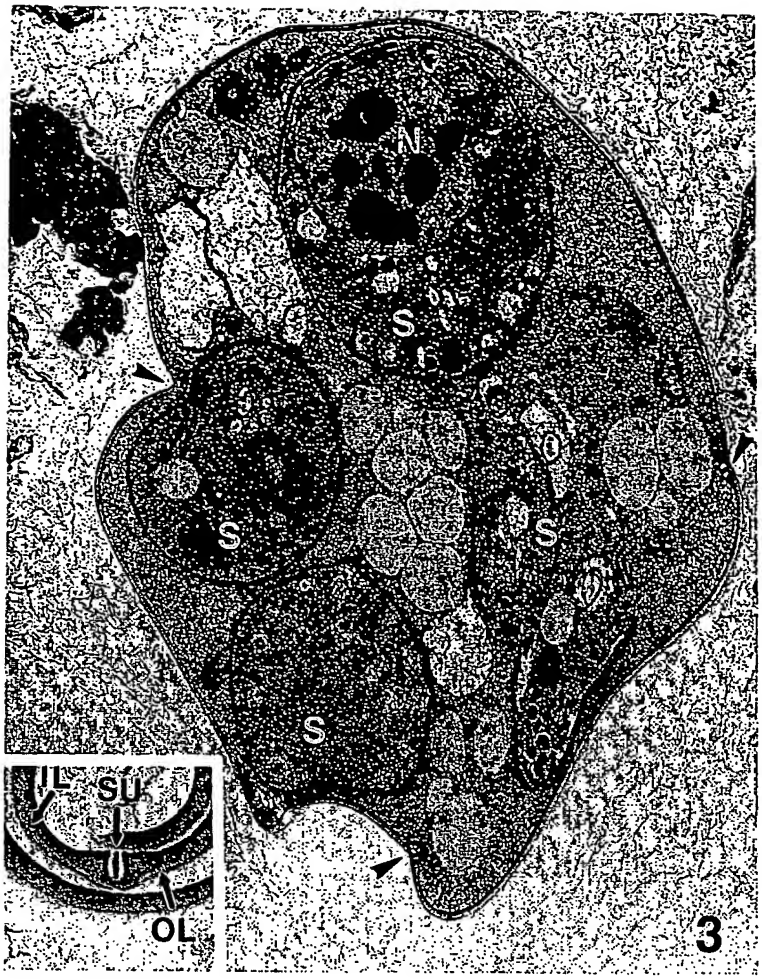


Fig. 3. Transmission electron micrograph of an *Isospora suis* sporocyst demonstrating four sporozoites (S) and three of four sutures (arrowheads). Note the nucleus (N) of one of the sporozoites. Magnification×13 600. Inset. Portion of a sporocyst wall demonstrating the inner layer (IL), outer layer and the suture (SU). Magnification×48 800.

of the mouse examined 4 h PI; only sporulated oocysts and free sporocysts were observed in the stomach. Sporulated oocysts, excysted sporocysts within oocysts, free sporocysts, and luminal sporozoites were observed in the duodenum, jejunum, and ileum of the mouse examined 6 h PI; sporulated oocysts and free sporocysts were observed in the stomach and sporulated oocysts, excysted sporocysts within oocysts and free sporocysts were observed in the cecum. Extraintestinal stages were not observed in the MLN of these mice.

Extraintestinal stages of *I. suis* were not observed in fresh smears of MLN or in tissue sections from any mice stained with hematoxylin and eosin, or

the ABPC immunohistochemical method (Table 1). Antibodies to *I. suis* were detected in the sera of BALB/c mice not treated with MPA (both 1:200) and in BALB/c mice treated with MPA (both 1:50). Sera from the four control mice and the *T. gondii* positive mouse were negative in the IFA test at a 1:50 dilution.

DISCUSSION

The structure of the oocyst wall of *I. suis* is similar to that reported for *Isospora canis* (Speer et al., 1973). Both have oocyst walls that are composed of three layers, the outer layer of which is altered by exposure to SH solution. A major difference is that the outer layer of the oocyst wall of *I. suis* is separated from the inner layer by a double unit membrane complex, whereas the outer layer of the oocyst wall of *I. canis* is separated from the inner layer by knob-like thickenings of the inner layer (Speer et al., 1973). Additionally, the outer layer of the oocyst wall of *I. suis* is electron-dense while that of *I. canis* is electron-lucent. The inner layer of *I. suis* oocyst walls is relatively uniform in appearance, and does not have the knob-like thickenings seen in the inner layer of *I. canis* oocysts (Speer et al., 1973). The oocyst wall of *T. gondii* has five layers (Christie et al., 1978) which makes it structurally different from *I. suis* and *I. canis*.

The structure of *I. suis* sporocysts and the excystation of sporozoites is similar to that described for other *Isospora* species (Speer et al., 1973, 1976), *Sarcocystis* species (Box et al., 1980; Strohlein and Prestwood, 1986) and *T. gondii* (Christie et al., 1978). One striking difference is in the number of layers present in the sporocyst wall. *Isospora canis*, *Isospora endocallimici*, and *I. suis* have two layers in the sporocyst wall while *T. gondii* has five layers and *Sarcocystis* species have three or more layers. Sporocysts of all these protozoans excyst by collapse of four separate curved sporocyst wall plates. The structure of extracellular sporozoites of *I. suis* observed in this study were identical to intracellular sporozoites observed in cell cultures (Lindsay et al., 1991).

Stuart et al. (1982) were not able to demonstrate extraintestinal stages of *I. suis* in mice orally inoculated with oocysts. They fed mice tissues to weaned pigs but failed to produce oocyst production consistently and had control pigs that became positive. They concluded that extraintestinal stages were not present in mice. We used outbred and inbred strains of mice, MPA treatment, and ABPC immunohistological staining to search for extraintestinal stages of *I. suis* in mice. However, we did not demonstrate stages of *I. suis* in the extraintestinal tissues of any mice examined in our study.

Results of our excystation studies in mice indicated that excystation occurs throughout the small intestine. Therefore, the inability of *I. suis* to excyst in mice is not an explanation for the absence of extraintestinal stages. It is inter-

esting that BALB/c mice orally inoculated with *I. suis* oocysts had serum antibodies to sporozoites when examined in the IFA test. These antibodies likely represent an immune response to antigens released from excysted oocysts in the intestine. However, they may represent an immune response to sporozoites that have penetrated host cells in some unidentified tissue. If the latter explanation is true, it would appear that the sporozoites are quickly destroyed by the host's immune system, because they were not demonstrable in tissues by the methods used in the present study.

We believe that sporocyst structure and the excystation process correlate with isosporan life cycles as suggested by Box et al. (1980). However, exceptions such as *I. suis* appear to exist. Additional studies with other mammalian *Isospora* species must be conducted to better define the presence and importance of extraintestinal stages of mammalian *Isospora* species that lack Stieda bodies in their sporocysts.

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